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## **Diagnostic and Protective Aspects of Humoral Immune Responses to Epstein-Barr Virus Encoded Proteins in Nasopharyngeal Carcinoma**

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EBV is a human oncogenic gammaherpesvirus, which has capacity to drive B cell growth-transformation. EBV infects >90% of the world population and mostly asymptomatic. EBV is transmitted via saliva and primary infection will be followed by lifelong latent infection of memory B lymphocytes and intermittent productive infection in sporadic nasopharyngeal mucosal epithelial cells. In adolescent, primary EBV infection may cause infectious mononucleosis (IM). EBV is associated with a variety of lymphoid and epithelial malignancies, including Burkitt's lymphoma, B-cell non-Hodgkin lymphomas in immunocompromised patients, Hodgkin's disease, T/Natural Killer (NK)-cell lymphomas, gastric adenocarcinomas and nasopharyngeal carcinoma. Different sets of EBV latent genes are expressed in the tumor cells of these malignancies.

Nasopharyngeal carcinoma (NPC) is an epithelial tumor of nasopharynx, and in particular the WHO III type is 100% associated with EBV. EBV+ NPC is a highly prevalent cancer in SE-Asia and ranks #4 in Indonesia, with 12.000 new cases diagnosed each year. The link between EBV infection and NPC was characterized by aberrant antibody responses to EBV, in particular by the elevation of IgG and IgA antibody titers to the EBV viral capsid antigen (VCA) and early antigen (EA). Antibodies against these lytic proteins and the EBV nuclear antigens 1 (EBNA1) are important for the diagnosis of NPC.

Most NPC patients are diagnosed at late stage, due to the limited and non-specific signs and symptoms at early stage, with consequently leads to poor therapy result. Therefore screening for NPC at early stage is crucial. However, standard diagnosis for NPC is made by an invasive biopsy, which cannot be applied to "look healthy" people. Many studies have shown that NPC can be identified by increased IgG and especially IgA levels against EBNA1, VCA and EA in the serum/plasma of patients. In this thesis we analysed the molecular basis for these increased anti-EBV responses in NPC patients and developed an ELISA method detecting IgG and IgA against native EA proteins which shows a good combination of sensitivity and specificity for NPC diagnosis. This assay is proposed as confirmation assay for NPC screening at early stage.

NPC at early stage is sensitive to radiotherapy. Radiotherapy with concomitant chemotherapy has increased survival, especially at higher stage of NPC. Unfortunately, they can produce undesirable complications after treatment. Furthermore, the outcome of patients with advanced stage disease at diagnosis or relapsing after first-line therapy is poor. Thus, additional forms of effective, low-

toxicity treatment are warranted. Since NPC is almost universally associated with EBV, several promising studies addressed cellular immunotherapy with EBV-specific cytotoxic T lymphocytes (CTLs), producing clinical responses. However, there are some obstacles in the development of CTL-based strategy. Therefore, in this thesis we explore the possibility of using the antibody against protein expressed on the NPC tumor cell, such as LMP1 and LMP2, to mediate lysis of the tumor cells through CDC or ADCC.

In **chapter 1** an overview is given on the current state-of-art knowhow on EBV biology and immunology as well as currently available diagnostic and intervention procedures. In **chapter 2** we analysed in detail the molecular diversity of IgG and IgA responses against EBNA1 and the whole spectrum of lytic EBV proteins, aiming to define the immunodominant markers, which discriminate NPC from healthy EBV carriers. By using immunoblot-strips containing EBNA1 and EBV lytic antigens separated by molecular weight, we identified IgG and IgA responses against the individual EBV antigens in serum panels of Indonesian, Caucasian, and Chinese ethnical background.

All NPC patients irrespective of their ethnic background, had an aberrant antibody recognition pattern, compared with regional control subjects, already at early stage NPC. In general NPC patients have elevated responses to similar proteins recognized by normal healthy individual (EBNA1, VCA-p18 and occasional VCA-p40 and Zebra), but show strong additional responses to EA polypeptides p138 (BALF2), TK (BXLF1), DNase (BGLF5), p47/54 (BMRF1), and Zebra (BZLF1).

In **chapter 3** we evaluated a number of EBV proteins, defined as immunodominant markers for NPC diagnosis, such as EBNA1, VCA-p18, EAd-p47/54 and EAd-p138. We compared a commercial qualitative EBV RecombLine test containing EBNA1, VCA-p18, VCA-p23, EAd-p47/54 and EAd-p138 with the in-house standardized immunoblot assay developed in chapter 2. The data show that IgG reactivity to VCA-p18, -p23, and EBNA1 is a poor marker for NPC diagnosis, since healthy controls also have such reactivity. IgG reactivity against EAd-p47/54 and -p138, yielded combined sensitivity/specificity and PPV/NPV values of 92.6%/98.3% and 99.0%/88.1%, for diagnosing NPC. Although, IgG reactivity to EAd-p138 and -p47/54 was also predominant over VCA-p18/-p23 in most IM cases, because IM is rare disease in NPC risk regions, this may not have consequences for diagnostic serology, NPC showed significantly more EBV reactive IgA antibody (>80% positive) than controls (<10% positive), although being less broadly reactive and significantly less strong compared to IgG. In high incidence NPC regions with low incidence IM yet high prevalence of EBV infection, both RecombLine IgG and IgA

tests provide a useful alternative confirmation test for NPC diagnosis, in particular when using EA and EBNA1 as discriminators in IgG and IgA testing, respectively.

In **chapter 4** we further explored the diagnostic value of EBV-EA components in more detail, by analyzing a recombinant proteins and synthetic peptides, and EA-specific extracts prepared by differential salt extraction from induced HH514.c16 cells as reference. The structure of epitopes on EA proteins appeared important for antibody interaction in NPC patients because “native” EA proteins provided a better antigen than peptides or artificially expressed recombinant proteins. The low salt “native protein” extract reproducibly prepared from purified nuclei of EA-induced HH514 cells, devoid of VCA and EBNA reactivity, contain characteristic EA polypeptides, such as p47/54 (BMRF1), p138 (BALF2), p55-DNAse (BGLF5) and p65-TK (BXLf1). This provided the best antigen for NPC diagnosis, with high sensitivity (>85%) and specificity (>94%) in both IgG and IgA responses compare to the recombinant proteins and synthetic peptides. The reproducible and simple “native” EA nuclear extract developed in this study applied in ELISA may contribute to the existing panel of serological assays for early diagnosis and post-treatment monitoring in NPC.

In **chapter 5** we proposed a simple and economical two-step ELISA system for diagnosing NPC in high-risk populations. A peptide-based IgA [EBNA1 plus VCAp18] ELISA, developed in a recent PhD-study (J. Fachiroh, thesis VUmc 2009) was used as an initial screening test and the IgA-EA ELISA, developed in chapter 4, as a confirmation test. Routinely, EBV IgG immunoblot, as developed in chapter 2, was used as a standard confirmation test. For diagnosing NPC, the two-step ELISA approach increased the sensitivity and specificity from 85.4% to 96.7% and 90.1% to 98%, respectively, with PPV and NPV increasing from 78.7 and 93.9% to 97.3 and 97.5%, respectively, relative to the immunoblot confirmation system.

In order to increase the reliability of the tools for NPC screening we proposed circulating EBV DNA as potential marker for confirmation of sero-diagnostic risk stratification in mass surveys. In **Chapter 6** we evaluated the application of a real-time Light Cycler PCR test targeting a conserved region of the EBNA1 gene for quantitative measurement of EBV DNA. Whole blood LC-PCR using 99-bp quantified a significantly higher EBV load (85.9%) than the 213-bp PCR assay (72.5%), confirming earlier data showing that circulating EBV DNA is fragmented, probably deriving from apoptotic tumor cell fragments. Since only limiting number of NPC patients had elevated DNA load in whole blood, we conclude that circulating EBV DNA load quantification is of limited value for primary NPC diagnosis as well as

for screening confirmation. However, recent studies suggested that EBV DNA load may be useful for therapy monitoring.

Previous chapters have focussed on analyzing antibody reactivity to components of intracellular EBNA, and specially the lytic EA, and VCA complexes, and the use of the antibody reactivity to those proteins for NPC diagnosis. However, most EBV-related tumors that arise in immunocompetent individuals, such as Burkitt Lymphoma, Hodgkins Disease, T-/NK-cell non-Hodgkin Lymphoma and NPC, only express latent EBV genes in the tumor cells. In **chapter 7** we analyzed the antibody responses against the latent EBV encoded and tumor-associated proteins, EBNA1, BARF1, LMP1 and LMP2 and explored the protective function of antibody responses against those proteins, in particular to domains accessible on the (tumor) cell surface. Naturally, the humoral immune responses (IgG and IgA) to individual EBV encoded tumor-associated membrane proteins (i.e. LMP1, LMP2A) and BARF1 is very low or mostly undetectable, except for EBNA1 which is strongly recognised. In particular, the potentially extracellular “loop” domains of LMP1 and LMP2 seem rather non-immunogenic, as well as secreted BARF1 protein. On the other hand, immunization of rabbit using synthetic peptides of selected epitopes representing “putative extracellular domains” of those proteins give rise to the generation of specific antibodies, capable binding to the surface of viable latent EBV infected cells. In addition, the antibodies against these putative LMP1 and LMP2 extracellular domains can mediate complement-driven cytotoxicity (CDC) of LMP1 and LMP2 expressing cell lines. This opens options for inducing therapeutic antibodies in NPC patients that can target tumor associated antigen expressed on the surface of NPC tumor cells, either via loop-peptide vaccination (active immunization) or infusion of loop-specific antibodies (passive immunization).

In conclusion, the molecular diversity of IgG and IgA antibodies in NPC patients is clearly deviant from healthy regional EBV carriers and can be exploited to design novel diagnostic tests. The ELISA method developed in this thesis detecting IgG and IgA against native EA extract, provides a standardized, simple and economical assay for NPC serodiagnosis. The method, especially IgA-EA, is proposed as confirmation test for the cheap peptide-based EBNA1 and VCA-p18 test and can be used for detecting NPC at early stage. The use of this 2-step ELISA method shows greatly improved sensitivity and specificity over existing methods.

This thesis also explored the protective function of humoral immune responses, especially against EBV tumor associated antigen expressed on NPC tumor cells, LMP1 and LMP2. The low response against these proteins reflects evolutionary immune escape mechanisms and can be enhanced by artificial

induction (immunization), yielding antibodies, which are able to kill LMP1 and LMP2 expressing (tumor) cells through CDC or ADCC. Regular serological screening of high-risk individuals together with developments in vaccination and (immune)therapy might decrease late-stage NPC incidence and further improve the clinical outcome for NPC patients in endemic regions like Indonesia, where NPC still is highly prevalent.